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Bioorganic & Medicinal Chemistry Letters 7 (13):p1791-1796, 1997, ISSN: 0960-894X

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0022-3565/98/2861-0419$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 286, No. 1
Printed in U.S.A.

JPET 286:419–428, 1998

Novel Chemically Modified Oligonucleotides Provide Potent Inhibition of P-Glycoprotein Expression[1]

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Accepted for publication March 2, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

One major form of multiple drug resistance (MDR) to cancer therapeutic agents is mediated by overexpression of P-glycoprotein, a membrane ATPase that serves as a drug efflux pump. In humans, this protein is the product of the *MDR1* gene. We have used chemically modified antisense oligonucleotides to reduce expression of P-glycoprotein in multidrug-resistant fibroblasts and colon carcinoma cells. Although several types of oligonucleotides were tested, compounds having a phosphorothioate backbone and a methoxyethoxy (ME) group at the 2′ position of the ribose ring proved to have the greatest potency. Thus, phosphorothioate 2′-ME oligonucleotides directed against either the AUG codon region or the stop codon region of the MDR1 message produced substantial (50–70%) inhibition of P-glycoprotein expression at concentrations of ≤50 nM. In addition, such treatment resulted in augmented drug uptake as measured by flow cytometry. Unmodified phosphorothioate compounds of the same sequence were active only in the micromolar range. We also tested the ability of several potential delivery agents to enhance the pharmacological effectiveness of anti-MDR1 oligonucleotides. Both commercial Lipofectin, a well known liposomal tranfection agent, and a liposomal preparation based on a novel "facial amphiphile" were effective in enhancing their pharmacological effects of antisense oligonucleotides. A Starburst dendrimer, a type of cationic polymer, was also effective in oligonucleotide delivery. This report emphasizes that significant improvements in antisense pharmacology can be made through judicious use of both chemical modifications of oligonucleotides and appropriate delivery systems.

MDR remains a significant problem for cancer chemotherapy (Dalton, 1994; Leyland-Jones *et al.*, 1993). One common form of MDR involves increased expression of one or more members of a family of transmembrane ATPases that serve as drug efflux pumps (Bradley and Long, 1994; Licht *et al.*, 1994; Roninson, 1992). The human *MDR* gene family has two members, one of which (*MDR1*) codes for a membrane ATPase termed the P-glycoprotein that is responsible for resistance to cytotoxic drugs (Roninson, 1992). In highly resistant cells, P-glycoprotein message and protein levels can be many times greater than those in drug-sensitive counterparts. A modest degree of MDR is usually due to increased transcription and translation from the *MDR1* locus, whereas high levels of MDR are often associated with extensive gene amplification (Bradley and Ling, 1994; Roninson, 1992). The MDR phenotype can be affected by agents that competitively inhibit P-glycoprotein-dependent antitumor drug efflux (Kajiji *et al.*, 1994; Leyland-Jones *et al.*, 1993). However, it has been difficult to identify chemical inhibitors of the P-glycoprotein function that have acceptable toxicities in the clinic (Chabner and Wilson, 1991; Dalton *et al.*, 1995). An alternative approach to inhibition of MDR would be the use of nucleic acid technologies to regulate the expression of *MDR1* message and its product, the P-glycoprotein.

Antisense oligonucleotides can potentially be used to modulate the expression of genes responsible for malignancy or for resistance to therapy through effects on gene stability, transcription, message processing, message stability or translation (Crooke and Bennett, 1996; Helene and Toulme, 1990; Stein and Cheng, 1993; Wagner, 1994). In fact, a number of reports have demonstrated antisense effects on cancer-related genes both in cell culture (Citro *et al.*, 1992; Monia *et al.*, 1996; Saison-Behmoaras *et al.*, 1991; Szczylik *et al.*, 1991; Vaughn *et al.*, 1995) and in murine tumors or human tumor xenograft models (Dean and McKay, 1994; Ensoli *et al.*, 1994; Higgins *et al.*, 1993; Nesterova and Cho-Chung, 1995; Schwab *et al.*, 1994; Skorski *et al.*, 1995). Recently, antisense oligonucleotide-based therapeutics has also begun to move into the clinical arena (Agrawal, 1996; Tonkinson and Stein,

Received for publication September 12, 1997.
[1] This work was supported by National Institutes of Health Grant CA47044 (R.L.J.). All oligonucleotides were provided by ISIS Pharmaceuticals.

ABBREVIATIONS: MEM, minimum essential medium; MDR, multiple drug resistance; ME, methoxyethoxy; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate.

1996). It is important to note that most successful examples of inhibition of gene regulation by antisense oligonucleotides involve messages that are present at relatively low levels. There are only a few examples in the literature (Knecht and Loomis, 1987; Vaughn *et al.*, 1995) of the successful use of antisense approaches for inhibition of highly expressed messages from single genes or of messages from highly amplified genes, such as occurs in MDR. Nevertheless, there has been substantial interest in using antisense oligonucleotides, or the related ribozyme technology, to control MDR. Using transfected anti-MDR ribozyme-containing plasmids, several laboratories have reduced P-glycoprotein message and protein expression and levels of drug resistance (Kiehntopf *et al.*, 1994; Kobayashi *et al.*, 1994; Scanlon *et al.*, 1994). In addition, there are several reports concerning antisense oligonucleotide effects on MDR (Alahari *et al.*, 1996; Corrias and Tonini, 1992; Efferth and Volm, 1993; Jaroszewski *et al.*, 1990; Rivoltini *et al.*, 1990). In a recent study, we used phosphorothioate or 5′ cholesterol derivatized phosphorothioate oligonucleotides directed against the region flanking the AUG codon to selectively inhibit MDR1 message and P-glycoprotein expression in mouse cells stably transfected with human MDR1 (Alahari *et al.*, 1996). However, control of MDR using antisense is quite difficult; the high expression levels of MDR1 message and of P-glycoprotein, as well as the slow turnover of P-glycoprotein (Zhang and Ling, 1991), present formidable challenges for antisense technology.

In this report, we examined two approaches for enhancing the pharmacological effectiveness of anti-MDR1 oligonucleotides. First, because stability to nucleases in the biological milieu is a major concern for antisense therapeutics (Akhtar and Juliano, 1992; Stein and Cheng, 1993), we used modified phosphorothioate oligonucleotides with ME groups at the 2′ position of the ribose ring. Typically, 2′ modified oligonucleotides are much more resistant to exonucleases than are standard oligonucleotides; in addition, heteroduplexes of RNA with 2′ modified oligonucleotides have a higher melting temperature (McKay *et al.*, 1996; Milligan *et al.*, 1993; Monia *et al.*, 1996), and this is also likely to be an advantage in attaining antisense effects. Because fully 2′ modified oligonucleotides do not support RNase H-mediated degradation of mRNA/antisense oligonucleotide heteroduplexes (Kawasaki *et al.*, 1993), and because RNase H activity often is vital for antisense effects, we used chimeric or "gapped" oligonucleotides with 5 residues at the 5′ end and 5 at the 3′ end modified with ME groups on the 2′ ribose position, whereas 10 interior residues were standard phosphorothioates (fig. 1). These compounds should be fully capable of supporting RNaseH activity (Monia *et al.*, 1996). Our studies suggest that 2′-ME-modified gapped antisense oligonucleotides are substantially more potent than unmodified phosphorothioates in inhibiting P-glycoprotein expression.

In addition to nuclease sensitivity, a second major problem for antisense therapeutics is the efficient delivery of oligonucleotides to the cytoplasm and the nucleus, the subcellular sites where pharmacological effects can occur (Akhtar and Juliano, 1992; Leonetti *et al.*, 1991; Shoji *et al.*, 1991; Wagner, 1994). In most cases, significant antisense effects in cell culture models have been achieved only through use of accessory substances, such as cationic lipids, that increase cell uptake and intracellular delivery of the antisense compounds (Bennett *et al.*, 1992; Stull and Szoka, 1995). In this report,

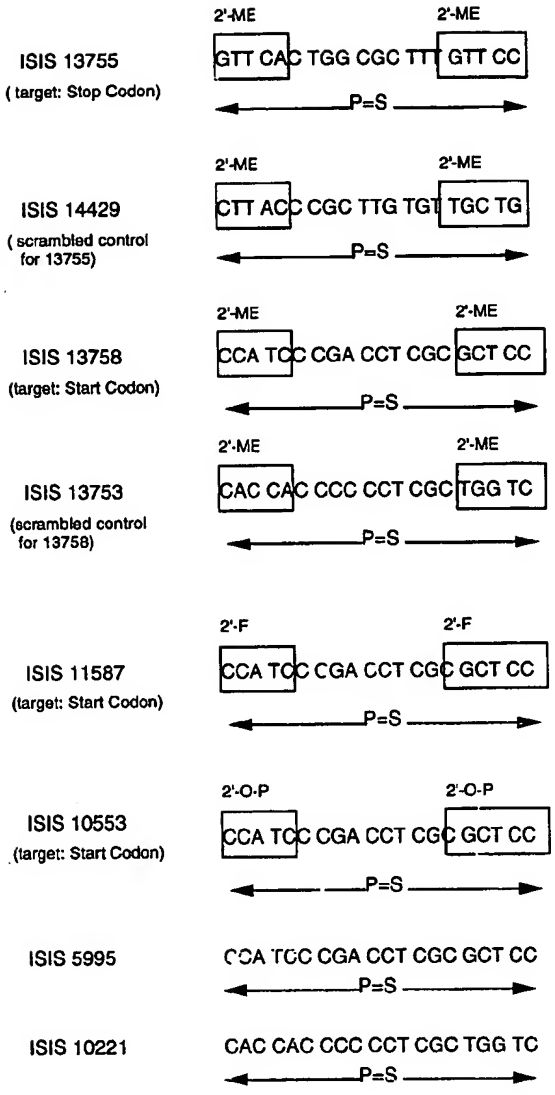

Fig. 1. Sequences and modifications of oligonucleotides. All oligonucleotides used had a phosphorothioate backbone. The areas enclosed in rectangles represent residues modified by substitution at the 2′ position with ME, propyl (P) or fluoro (F) substituents. The oligonucleotides targeted to the start codon and their scrambled controls all had the same sequences as previously described ISIS 5995 (antisense) and its control ISIS 10221 (Alahari *et al.*, 1996). The compound ISIS 13755 is targeted to a sequence flanking the stop codon, whereas ISIS 14429 is its scrambled control.

we examined several novel oligonucleotide delivery agents and have compared them with cationic liposomes (Lipofectin) (Felgner and Ringold, 1989) that have been widely used to promote DNA transfection and antisense oligonucleotide delivery (Stull and Szoka, 1995). Current studies indicate that several other delivery technologies may also be partially effective in enhancing the pharmacological actions of antisense oligonucleotides and that delivery of oligonucleotides to the nucleus is strongly correlated with pharmacological effect.

Experimental Procedures

Cells. NIH 3T3 cells transfected with a plasmid containing the human *MDR1* gene (*pSK1 MDR*), as well as the plasmid itself, were gifts from M. M. Gottesman (Kane *et al.*, 1989). The cells were grown

in DMEM containing 10% FCS and 60 ng/ml colchicine in an atmosphere of 95% air/5% CO_2. LoVo ADR 1.2, a subline of human colon carcinoma cells selected for resistance to doxorubicin, were also used (obtained from L. Y. Yang, M. D. Anderson Tumor Institute, Houston, TX). These cells were grown in Ham's F-10 containing 10% FBS and 1.2 μg/ml doxorubicin.

Oligonucleotide synthesis. Phosphorothioate oligonucleotides, as well as chimeric 2'-propyl, fluoro and methoxyethoxy phosphorothioate oligonucleotides, were synthesized at ISIS Pharmaceuticals using previously described procedures (Monia *et al.*, 1996). Oligonucleotide sequences are shown in figure 1. Additional phosphorothioate oligonucleotides and fluorophore-labeled phosphorothioate oligonucleotides were synthesized at the Oligonucleotide Core Facility (Lineberger Cancer Center, University of North Carolina). The fluorophores, fluorescein and cyanine-5 were added at the 5' terminus as amidites (Glen Research).

Delivery agents. Agents tested for their ability to enhance the cellular delivery and pharmacological effectiveness of anti-MDR oligonucleotides included (1) Lipofectin (BRL Laboratories), a commercial transfection agent composed of cationic liposomes containing dioleoyl phosphatidyl choline and the cationic amphiphile DOTMA (Felgner and Ringold, 1989); (2) Starburst dendrimers, generation 3 (Aldrich Chemical, Milwaukee, WI) and generation 7 (Life Sciences), are branched cationic polymers containing amino residues that have previously been reported to increase transfection efficiency of plasmids (Bielinska *et al.*, 1996; Haensler and Szoka, 1993) and to enhance cellular accumulation of oligonucleotides (DeLong *et al.*, 1997); (3) N-(5,10,14-triazatetradecyl)deoxycholic amide (also termed compound 7 below) obtained from S. Regen, Lehigh University (Sadownik *et al.*, 1995) (this is a so-called "facial amphiphile" similar to compounds previously reported to increase plasmid transfection efficiency when used as liposomal complexes; Walker *et al.*, 1996); and (4) GALA, an amphipathic 30-residue peptide (Parente *et al.*, 1990) that has previously been reported to increase transfection efficiency of plasmids (Plank *et al.*, 1994) as well as enhancing the pharmacological effects of antisense oligonucleotides (Hughes *et al.*, 1996).

Treatment of cells with oligonucleotides. The experimental protocols were similar to those we previously described (Alahari *et al.*, 1996). Briefly, MDR-3T3 cells were grown onto 162-mm flasks to 95% confluency and then seeded onto 100-mm dishes at 5×10^6/dish in 10% FCS/DMEM and incubated for 24 hr. The cells were washed twice with PBS. Oligonucleotides, in some cases accompanied by various delivery agents, were added in Opti-MEM and incubated at 37°C overnight. Thereafter, the cells were further incubated at 37°C in DMEM plus 2% FCS for 48 hr before harvesting for analysis.

The various delivery agents were used as follows. Lipofectin (20 μg/ml) and various amounts of oligonucleotide were mixed in Opti-MEM, preincubated at room temperature for 30 min and then added to the cells. Compound 7 was incorporated into liposomes by mixing 5 mg of compound 7 and 5 mg of dioleoyl phosphatidyl choline (Avanti Polar Lipids, Birmingham, AL) in chloroform/methanol, drying the mixture in a rotary evaporator and then dispersing in sterile water to form liposomes. The compound 7 liposomes were then complexed with oligonucleotides and used to treat cells in the same manner as Lipofectin. Dendrimers were prepared as a stock solution of 1 mM in water; dendrimer/oligonucleotide complexes of ~10:1 molar ratio (dendrimer/oligonucleotide) were formed at positive charge excess by adding 20 μl of dendrimer to a 10-μl sample of oligonucleotide in water. The dendrimer/oligonucleotide complexes were then used to treat cells. The GALA amphipathic peptide was dissolved in PBS and preincubated with cells for 30 min at 200 μg/ml before the addition of oligonucleotides and continued incubation.

The cytotoxicity of the various treatments used in the oligonucleotide experiments was evaluated in preliminary experiments by using a vital dye assay. Conditions were chosen such that there was usually <10% difference in the number of viable cells in samples treated with oligonucleotides and delivery agents *vs.* control samples maintained in medium alone; exceptions are noted in the text. The MDR-3T3 cells maintained a high level of viability during extended incubation in serum-free Opti-MEM, but cell division was largely suppressed.

Measurement of MDR1 mRNA expression by Northern blotting. Measurement of the effects of oligonucleotides on levels of MDR1 mRNA were conducted essentially as we previously described (Alahari *et al.*, 1996). Briefly, total cellular RNA was isolated by lysis in 4 M guanidium isothiocyanate followed by a cesium chloride gradient, and the RNA was resolved on 1.2% agarose gels containing 1.2% formaldehyde and transferred to nitrocellulose membranes. The blots were hybridized with a ^{32}P-radiolabeled human MDR1 cDNA probe that was prepared by performing a polymerase chain reaction on the pSK1 MDR plasmid. The filters were hybridized overnight in hybridization buffer (25 mM KPO_4, pH.7.4; 5× SSC, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA and 50% formamide). This was followed by two washes with 1× SSC + 0.1% SDS and two washes with 0.25× SSC + 0.1% SDS. Hybridizing bands were visualized by exposure to X-OMAT AR film and quantified using a PhosphorImager (Molecular Dynamics, Sennyvale, CA). To confirm equal loading of RNA, the blots were stripped and reprobed with a ^{32}P-labeled actin probe (Clontech, Palo Alto, CA).

Measurement of P-glycoprotein expression by Western blotting. The effect of oligonucleotides on total P-glycoprotein expression was measured by Western blotting as previously described (Alahari *et al.*, 1996). The protein content of cell lysates was determined using the bicinchonic acid assay (Pierce Chemical, Rockford, IL) according to the manufacturer's directions. Equal amounts of cell protein were loaded onto each gel lane. The C219 anti-P-glycoprotein antibody (Signet, Dedham, MA) was used as a primary antibody. Immunoreactive proteins were visualized either by enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, IL) or with ^{125}I secondary antibodies.

Measurement of cell surface P-glycoprotein by flow cytometry. The effects of oligonucleotides on cell surface expression of P-glycoprotein were determined using a flow cytometry assay as we previously described (Alahari *et al.*, 1996). The MRK16 anti-P-glycoprotein antibody (Kamiya Biochemicals, Thousand Oaks, CA), which is directed against an external epitope, was used as the primary antibody. An R-PE-conjugated goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) was used as the second antibody. The level of R-PE fluorescence in viable cells (as determined by light scatter) was quantified using the Cicero software application (Cytomation, Fort Collins, CO) on a Becton Dickinson flow cytometer.

Measurement of cellular accumulation and intracellular distribution of fluorophore-labeled oligonucleotides. The total cellular accumulation of fluorescein isothiocyanate-labeled phosphorothioate oligonucleotide (FITC-5995) in viable cells was quantified by flow cytometry. The incubation conditions were usually 0.5 to 1.0 μM fluorescent oligonucleotide in serum-free DMEM or Opti-MEM for 12 to 15 hr at 37°C. The cellular uptake and intracellular distribution of fluorescent oligonucleotides were visualized in viable cells using a Leica TCS 4D confocal microscope with a plan/apo 1.4-NA oil objective (40×). The incubation conditions were the same as for the flow cytometry experiments; however, a cyanine-labeled phosphorothioate oligonucleotide based on the 5995 sequence was used in confocal microscopy studies because there was virtually no background autofluorescence from the cells at the relatively long (667 nm) emission wavelength used to visualize this fluorophore. The cell surface was visualized by counterstaining the cells (after incubation with oligonucleotides) with the MRK16 anti-P-glycoprotein antibody, followed by an FITC-labeled goat anti-mouse antibody (Sigma Immunochemicals).

Measurement of rhodamine 123 uptake by flow cytometry. We examined cell uptake of the fluorescent dye rhodamine 123 as a surrogate for uptake of antitumor drug; this approach has been widely used in the multidrug-resistance field (Twentyman *et al.*, 1994). The procedure was essentially that described by Alahari *et al.*

(1996) with minor changes. Briefly, 5×10^6 cells were seeded onto six-well plates, incubated for 1 day and treated with 10 nM oligonucleotides as described above. Cells were removed with pancreatin and resuspended in 10% FBS/DMEM. Rhodamine 123 (Sigma Chemical) was dissolved in water, added to a final concentration of 1.0 μg/ml and incubated for 1 hr at 37°C; 500-μl samples were taken at several points, washed with medium once and resuspended in 500 μl of medium. Viable cells were analyzed for the accumulation of rhodamine 123 on a Becton Dickinson flow cytometer using Cicero software.

Results

Characteristics of the anti-MDR1 oligonucleotides tested. The oligonucleotides used in this study are shown in figure 1. Oligonucleotides 5995 and 10221 have been previously described (Alahari *et al.*, 1996) and are unmodified phosphorothioates; 5995 is directed to the AUG codon region (positions 409–428) of the MDR1 message, whereas 10221 is its scrambled control. Oligonucleotide 13758 is a 2′-ME-phosphorothioate chimera directed at the AUG codon region with the same sequence as 5995 (positions 409–428), and 13753 is its scrambled control. Oligonucleotide 13755 is a 2′-ME-phosphorothioate chimera directed to the stop codon region of the MDR1 message (positions 4250–4269), and 14429 is its scrambled control. In addition, 11587 and 10553 are 2′-fluoro-phosphorothioate and 2′-O-propyl phosphorothioate chimeras, respectively, with the same sequence as 13758 (targeted to AUG).

Inhibition of cell surface expression of P-glycoprotein. The effect of anti-MDR1 methoxyethoxy-phosphorothioate oligonucleotides on cell surface expression of P-glycoprotein in MDR-3T3 cells was evaluated using immunofluorescence and flow cytometry. In these initial experiments, Lipofectin was used as a delivery agent. As seen in figure 2, treatment with 100 nM concentrations of the 2′-ME oligonucleotides 13755 or 13758, but not treatment with scrambled control, resulted in major reductions in the fluorescence intensity of cells staining with anti-PGP antibody. Thus, both the antisense oligonucleotide directed against the AUG codon (13755) and the antisense oligonucleotide directed against the stop codon (13758) had substantial effects. The observed effects using 100 nM concentrations of ME oligonucleotides were as great as those previously observed using micromolar concentrations of unmodified phosphorothioate oligonucleotides, suggesting that the ME oligonucleotides were more potent.

This issue was further evaluated by performing a dose-response analysis for the ME oligonucleotides using either MDR-3T3 cells (fig. 3A) or LoVo cells (fig. 3B). As seen in figure 3A, compound 13758 caused a 50% reduction in cell surface PGP levels in 3T3 cells at concentrations as low as 10 nM, whereas 13755 attained a 50% reduction at 50 nM. As seen in figure 3B, the LoVo cells were somewhat less sensitive, with a 40% reduction in PGP expression attained at 100 nM for both 13755 and 13758. These observations contrast with previous observations using compound 5995, an unmodified phosphorothioate antisense oligonucleotide directed at the MDR1 AUG codon, where 40% to 50% specific antisense inhibition of cell surface expression was attained only at 1 to 2 μM concentrations (Alahari *et al.*, 1996).

A direct comparison of several types of anti-MDR1 antisense oligonucleotides, each directed against the AUG codon,

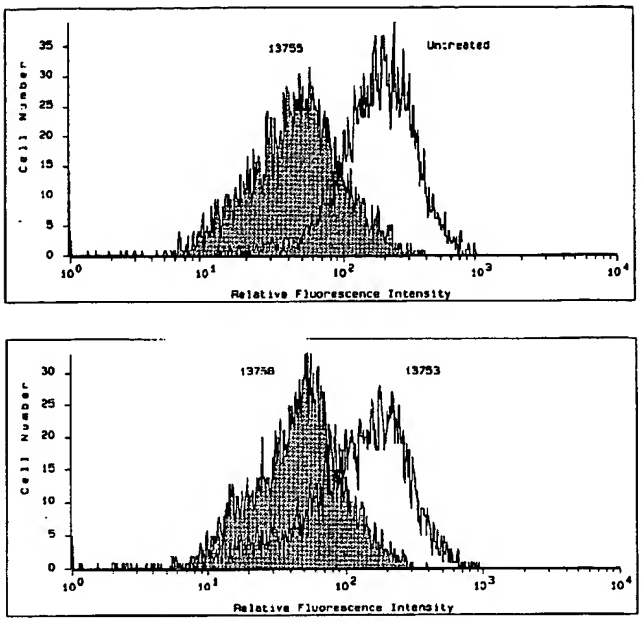

Fig. 2. Effect of ME oligonucleotides on P-glycoprotein cell surface expression. MDR-3T3 cells were maintained as untreated controls or treated with 100 nM concentrations of ISIS 13755 (targeted to stop codon), ISIS 13758 (targeted to AUG, analog of 5995) or ISIS 13753 (scrambled control, analog of 10221) in Opti-MEM with Lipofectin overnight and then incubated for an additional 48 hr in 2% FBS containing DMEM. The cells were stained with anti-PGP antibody MRK 16, followed by R-phycoerythrin-conjugated secondary antibody. The traces show cell number on the *y*-axis *vs.* relative phycoerythrin fluorescence on the *x*-axis.

is given in figure 4. In this experiment, a concentration of 100 nM, the unmodified phosphorothioate 5995, resulted in a 30% inhibition of PGP expression; the 2′-O-propyl chimera 10553, a 50% inhibition; the 2′-fluoro-chimera 11587, a 60% inhibition; and the ME-chimera 13758, a 70% inhibition. We noted among-experiment quantitative variation in the degree of inhibition of PGP expression caused by the various antisense oligonucleotides; however, the ME oligonucleotides consistently provided the greatest inhibition at any given concentration of oligonucleotide.

Reduction in MDR1 message and protein levels. To determine whether the observed inhibition of cell surface P-glycoprotein expression was correlated with a reduction in message levels, we performed Northern analysis of total RNA extracted from control or oligonucleotide-treated MDR-3T3 cells. The MDR1 message was detected with a 1.0-kb MDR1 probe; the blots were then stripped and reprobed for β-actin message, and the ratio of the two messages was quantified using a PhosphorImager, as we have previously described (Alahari *et al.*, 1996). In a typical experiment (fig. 5, A and B), treatment of cells with the ME oligonucleotides 13758 (AUG codon) and 13755 (stop codon) resulted in significant (~50%) reductions in the MDR/actin message ratios, whereas treatment with the scrambled control oligonucleotide 13753 had little effect. Antisense oligonucleotide-mediated reduction in mRNA levels is often attributed to RNaseH activity (Helene and Toulme, 1990). Thus, it seems likely that the 13758 and 13755 oligonucleotides exert much of their effect on P-glycoprotein expression through degradation of its messenger RNA. Figure 5C shows the effect of oligonucleotide treatment

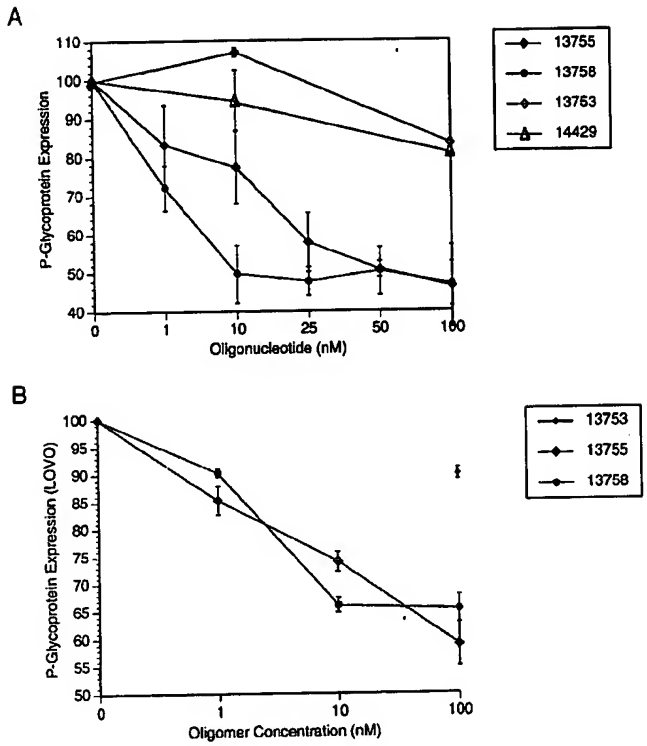

Fig. 3. Dose response for ME oligonucleotide inhibition of P-glycoprotein cell surface expression. A, MDR-3T3 cells were treated with various concentrations of ISIS 13753, 13755, 13758 or 14429 oligonucleotides overnight in Opti-MEM and incubated 48 hr further in 2% FBS/DMEM. Cells were stained with anti-PGP monoclonal antibody as described above. Data are presented as percentage inhibition of P-glycoprotein expression, with the 100% level taken as that for untreated MDR-3T3 cells. The results represent mean and S.D. values of four determinations. B, ADR-resistant LoVo colon cancer cells were treated in the same manner as in A. Values are mean and S.D. of three determinations.

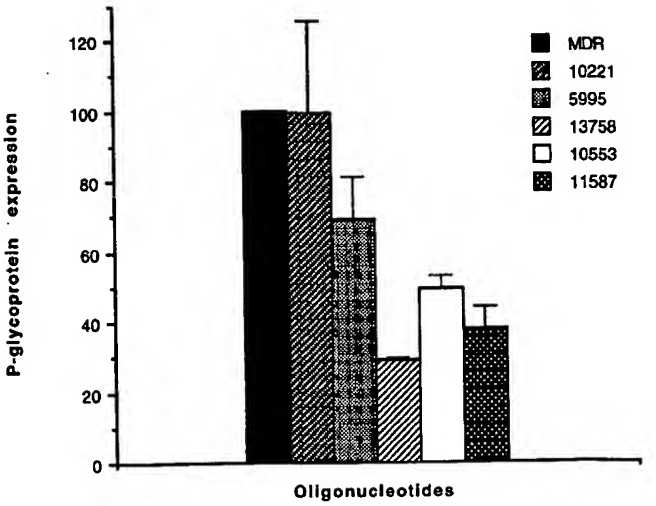

Fig. 4. Comparison of inhibitory effects of different chemically modified oligonucleotides. Inhibition of P-glycoprotein expression by unmodified phosphorothioate oligonucleotides (5995) or phosphorothioate oligonucleotides with 2'-O-ME (13758), 2'-O-propyl (10553) or 2'-O-fluoro (11587) modifications. All oligonucleotides were targeted to the AUG codon, except that 10221 is a scrambled control. MDR-3T3 cells were treated with oligonucleotides and analyzed for cell surface expression of P-glycoprotein as described in figure 2. Data are presented as percent of control with the 100% level taken as that for untreated MDR cells. Values are mean and S.D. for three separate experiments.

on total P-glycoprotein levels as visualized by Western blotting equal amounts of cell lysate protein using the c219 antibody. Both 13758 (AUG codon) and 13755 (stop codon) produced reductions in the level of total cellular P-glycoprotein similar to the reduced cell surface display demonstrated in figures 2 and 3.

Effects of anti-MDR1 antisense oligonucleotides on drug uptake. Rhodamine 123 uptake is a commonly used surrogate for uptake of clinically useful anticancer drugs because uptake of this fluorescent compound can readily be measured by flow cytometry (Twentyman *et al.*, 1994). As seen in figure 6, treatment with the antisense oligonucleotides 13758 or with 13755, but not with control oligonucleotides, elicited a modest but significant increase in uptake of rhodamine 123 by the drug-resistant cells. The magnitude of the effect, ~1.5- to 2.0 fold, is similar in magnitude to the change in P-glycoprotein expression as measured by Western blotting (fig. 5C). The rhodamine 123 uptake assay provides a more sensitive indication of changes in drug transport than assays based on cytotoxicity.

Effects of delivery agents ("adjuvants") on the uptake, subcellular distribution and effectiveness of anti-MDR1 oligonucleotides. We have been interested in devising approaches to improve the delivery of antisense oligonucleotides to their intracellular targets (Akhtar and Juliano, 1992; Hughes *et al.*, 1996). In this report, we compare the effects of several novel agents ("adjuvants"; see Hughes *et al.* 1996) with that of Lipofectin, a cationic lipid preparation that has been widely used for plasmid transfection and oligonucleotide delivery (Stull and Szoka, 1995). The agents tested were an amphipathic peptide termed GALA, generation 3 and 7 cationic dendrimers, a cationic lipid complex composed of dioleoyl phosphatidyl choline and a positively charged "facial amphiphile" (compound 7). Although each of these substances has the potential to cause cytotoxicity, the amounts used were chosen so as to result in minimal toxicity as judged by cell loss and changes in cell morphology (Hughes *et al.*, 1996). In an initial set of experiments, we used a flow cytometry assay to examine the ability of the various "adjuvants" to increase the total amount of fluorophore-labeled oligonucleotide that became cell associated. As seen in figure 7, treatment with the GALA peptide had no effect on cell accumulation of oligonucleotide, whereas cationic dendrimer, the facial amphiphile complex and commercial Lipofectin all caused major increases in cell association, with Lipofectin giving the greatest increase.

To further explore the actions of these potential delivery agents, we examined their effects on the subcellular distribution of fluorescent oligonucleotides by using confocal microscopy (fig. 8). The cells were simultaneously imaged for oligonucleotide (red channel) and for expression of P-glycoprotein (green channel); the anti-P-glycoprotein immunostaining allowed ready visualization of the shape of the cells (the conditions used in these imaging experiments were not designed to result in inhibition of expression of P-glycoprotein). As seen in figure 8, exposure to oligonucleotide alone (fig. 8A) or to oligonucleotide plus GALA peptide (fig. 8B) resulted in low levels of oligonucleotide accumulation in cells and little nuclear accumulation. By contrast, cells treated with oligonucleotide plus the facial amphiphile complex (fig. 8C) or

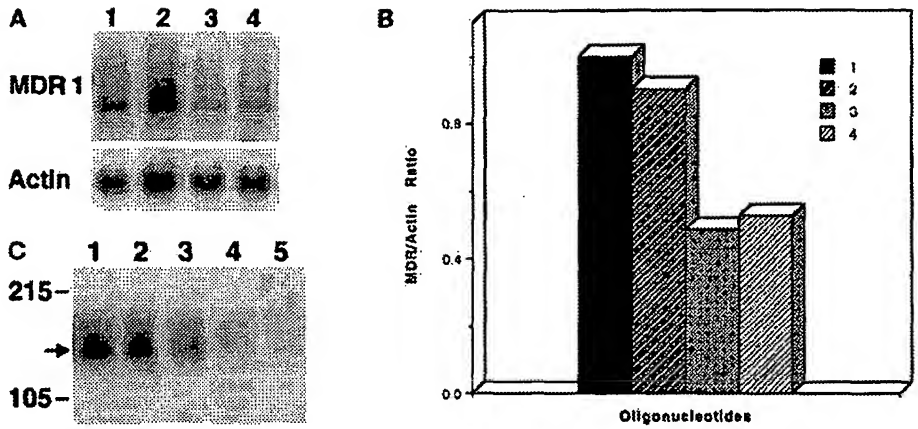

Fig. 5. Effect of ME oligonucleotides on MDR1 message and protein expression. MDR-3T3 cells were grown to 90% confluence and treated with 100 nM oligonucleotides overnight in the presence of Lipofectin in Opti-MEM and for an additional 24 hr in 2% serum-containing medium. A, Northern blots. Total RNA was isolated, fractionated on agarose formaldehyde gels and blotted onto nitrocellulose membranes as described in the text. These membranes were probed with a ^{32}P-radiolabeled 1.0-kb MDR-1 cDNA (top) and then stripped and reprobed with a ^{32}P-radiolabeled β-actin cDNA(bottom). Lane 1, untreated MDR-3T3 cells. Lane 2, 13753, scrambled control. Lane 3, 13755, targeted to the stop codon. Lane 4, 13758, targeted to the start codon. B, Quantification of Northern blots using a PhosphorImager; the ratio of MDR1 message to actin message was calculated. Column 1, untreated control; column 2, 13753, scrambled control; column 3, 13755, targeted to the stop codon; lane 4, 13758, targeted to the start codon. C, Western blots. Cell lysates were prepared as described in the text, and the total protein content was determined. Equal amounts of cell lysate protein (10 μg) of were run on 8% SDS-PAGE gels. Total cellular P-glycoprotein was detected by Western blotting with c219 monoclonal antibody followed by detection using enhanced chemiluminescence. Lane 1, untreated MDR-3T3 cells. Lane 2, 14429, scrambled control. Lane 3, 13753, scrambled control. Lane 4, 13755, targeted to the stop codon. Lane 5, 13758, targeted to the start codon.

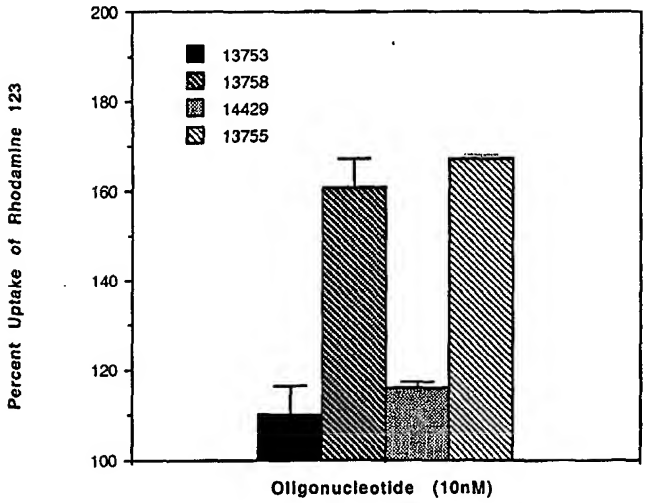

Fig. 6. Cell uptake of rhodamine 123 by control and antisense treated cells. MDR-NHIH 3T3 cells were treated with 10 nM antisense (13758, 13755) or control oligonucleotides (13753, 14429) as described above. After a 24-hr pretreatment with oligonucleotides, the cells were exposed to 1 μg/ml rhodamine 123 fluorophore for 60 min at 37°C. Cells were rinsed once with complete medium and then analyzed in a Becton Dickinson flow cytometer for uptake of the fluorophore. Results are the mean and S.D. values of triplicate determinations and are normalized to untreated control MDR-3T3 cells as 100%.

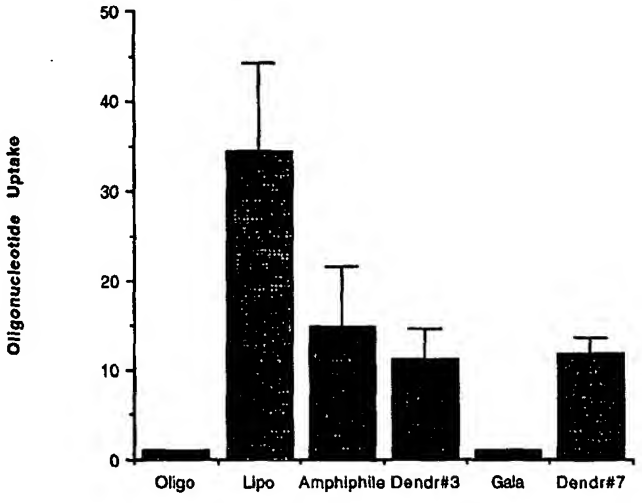

Fig. 7. Cell uptake of oligonucleotides promoted by delivery agents. A fluorescein (FITC)-labeled oligonucleotide with the same sequence as 5995 was complexed with various potential delivery agents ("adjuvants'). Then, 100 nmol of oligonucleotide was mixed with 20 μg/ml Lipofectin, 25 μg/ml of facial amphiphile liposome complexes or 10 uM dendrimer, incubated for 20 minutes at room temperature and added to MDR-3T3 cells in 1 ml of Opti-MEM. In the case of the GALA peptide, 200 μg/ml was used with 100 nmol of oligonucleotides. After a 15-hr incubation, the amount of cell-associated fluorescent oligonucleotide was quantified by flow cytometry as described in the text. Results are given as the ratio of uptake in samples treated with "adjuvants' compared with cells exposed to free oligonucleotides (free = 1) and are the mean and S.D. values of six determinations.

Lipofectin (fig. 8D) displayed extensive cell uptake, with many cells showing dramatic nuclear staining. The use of the generation 3 dendrimer or of dendrimer plus GALA resulted in a moderate increase in cell accumulation but little nuclear staining (not shown). Use of the generation 7 dendrimer resulted in nuclear accumulation of oligonucleotide in a significant number of cells (not shown). A series of microscopic fields, each averaging ~50 cells, were scored for the percentage of cells displaying obvious nuclear fluorescence. The mean and S.D. values were free oligonucleotide, 0%; GALA peptide, 5 ± 2.5%; facial amphiphile complex, 52 ± 5%; Lipofectin, 60 ± 5%; and generation 7 dendrimer, 40 ± 4%.

The impact of the various adjuvants on the pharmacological effectiveness of anti-MDR1 oligonucleotides was evaluated by measuring the cell surface expression of P-glycoprotein using a flow cytometry assay. As seen in figure 9, treatments using scrambled ME oligonucleotide 13753

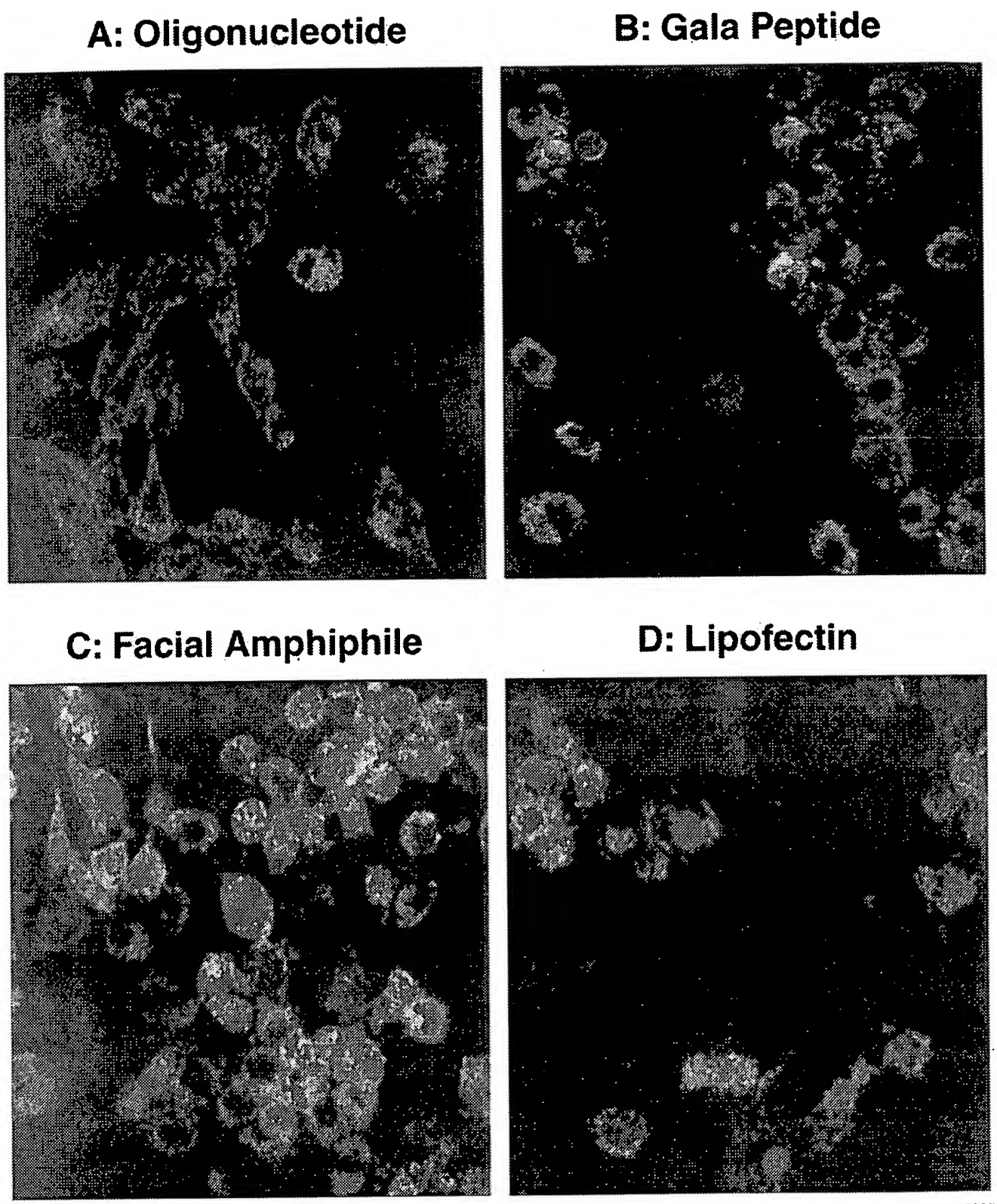

Fig. 8. Subcellular localization of oligonucleotides promoted by delivery agents. A cyanine labeled oligonucleotide with the same sequence as 5995 was complexed with various potential delivery agents ("adjuvants') as described in figure 7. MDR-3T3 cells were cultured on glass coverslips and then exposed to the fluorescent oligonucleotide, with or without the "adjuvant" for 15 hr in Opti-MEM. The cells were washed, briefly incubated in complete medium and then counterstained with the MRK 16 anti P-glycoprotein antibody, followed by an FITC-labeled second antibody. Fluorescence was visualized by confocal microscopy, as described in the text. The oligonucleotide is visualized in orange, and the P-glycoprotein surface staining is visualized in green. A, Free oligonucleotide. B, Oligonucleotide plus GALA peptide. C, Oligonucleotide plus facial amphiphile/liposome complexes. D, Oligonucleotide plus Lipofectin.

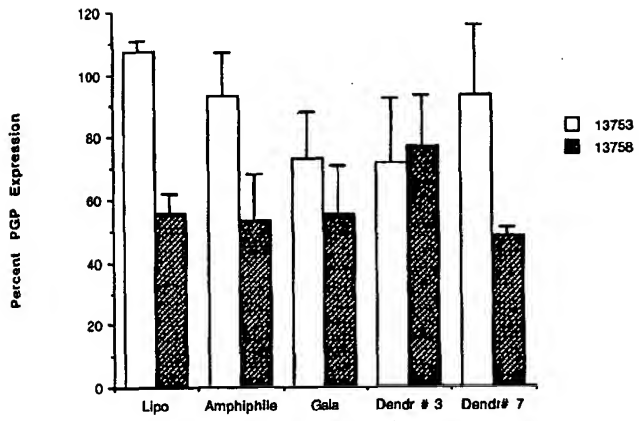

Fig. 9. Pharmacological effects of delivery approaches. ME-modified oligonucleotides 13758 (antisense, start codon) and 13753 (scrambled control) were complexed with various delivery agents. Then, 100 nmol of oligonucleotides was mixed with 20 μg/ml Lipofectin, 25 μg/ml of facial amphiphile liposome complexes or 10 μM dendrimer; incubated for 20 min at room temperature; and added to cells in 1 ml of Opti-MEM medium in six-well culture dishes. In the case of the GALA peptide, 200 μg/ml was used with 100 nmol of oligonucleotides. After overnight incubation, the medium was replaced by 2% serum-containing medium, and the cells were incubated further for 2 days. Flow cytometry analysis for P-glycoprotein expression was done as described in figure 1.

caused slight reductions in P-glycoprotein expression compared with untreated controls; these probably represent minor nonspecific cytotoxic effects. Treatment using ME oligonucleotide 13758 (AUG codon) and the adjuvants Lipofectin or the facial amphiphile complex resulted in substantial reductions (40–50%) in P-glycoprotein expression compared with the scrambled controls; this was also true of the generation 7 dendrimer. These results were statistically significant at $P < .001$. The use of the generation 3 dendrimer failed to produce a reduction in P-glycoprotein expression, whereas the GALA peptide tended to provide a greater reduction than antisense oligonucleotide alone, but the result was not statistically significant.

Discussion

Antisense oligonucleotide technology is increasingly becoming a reliable tool for manipulation of gene expression in laboratory situations and is rapidly moving into the therapeutic arena (Agrawal, 1996; Crooke and Bennett, 1996). The future progress of antisense based therapeutics will require oligonucleotides that are stable in the biological milieu, have a strong and specific association with their mRNA targets and have the ability to trigger enzymatic RNA degradation processes. The chimeric 2′-ME- phosphorothioate oligonucleotides described here represent a significant step toward this ideal. As demonstrated above, ME oligonucleotides directed against either the AUG codon (13758) or the stop codon (13755) of the MDR1 message can cause substantial reductions in both P-glycoprotein expression and message levels. Maximal effects are observed in the range of 10 to 50 nM; this degree of potency is significantly greater than that displayed by an unmodified phosphorothioate (5995) directed against the same sequence, where major effects are seen only in the micromolar range. In this study, we have not elucidated the biochemical basis of the greater potency of the chimeric 2′-ME oligonucleotides; however, previous investigations of chemically modified chimeric oligonucleotides have suggested that nuclease resistance, and thus the concentration and duration of the antisense oligonucleotide within the cell, plays a key role in pharmacological efficacy (Monia *et al.*, 1996). Our current results with 2′-ME oligonucleotides suggests that significant improvements in the pharmacological effects of antisense compounds can continue to be made through innovative modifications of their chemistry. However, the approximate 2-fold reductions in P-glycoprotein expression observed here in response to antisense treatment may not be sufficient to be of significant therapeutic value. Nevertheless, given the fact that the highly overexpressed and very stable *MDR1* gene product is a challenging target for antisense modulation, current results are encouraging. In particular, our results suggest (see below) that the major obstacle to attaining complete MDR inhibition is inefficient delivery of the antisense oligonucleotides to the entire cell population and that attaining more homogeneous delivery could result in greatly improved pharmacological effect.

Thus, in addition to the chemical characteristics of the antisense oligonucleotides used, another key aspect of antisense therapeutics involves the effective delivery of oligonucleotides into the cytoplasmic and nuclear compartments (Akhtar and Juliano, 1992). In the *in vivo* setting, antisense effects have been observed on the administration of antisense oligonucleotides in free form (Dean and McKay, 1994; Nesterova and Cho-Chung, 1995; Skorski *et al.*, 1995). However, in cell culture models, use of antisense compounds complexed with a delivery agent usually seems to be required (Bennett *et al.*, 1992; Stull and Szoka, 1995). The reason for the apparent discrepancy between the *in vivo* and *in vitro* situations is unclear at this point; furthermore, it remains to be seen whether various delivery agents might enhance *in vivo* antisense effects.

In *in vitro* studies, there is a good correlation between nuclear delivery of oligonucleotides and pharmacological effect. In our studies, the "adjuvants" Lipofectin, the facial amphiphile complex and the generation 7 dendrimer provided the best delivery of oligonucleotide to the nucleus and the greatest enhancement of pharmacological effects. However, even using Lipofectin as a delivery agent, in various experiments we were unable to attain more than 50% to 70% inhibition of P-glycoprotein expression with highly potent chimeric 2′-ME oligonucleotides. We believe that this is due to the heterogeneous delivery attained with Lipofectin and other cationic lipid complexes, rather than an inability of the chimeric 2′-ME oligonucleotides to fully suppress message levels in those cells where it reaches the nucleus. This concept is supported by confocal microscopic observations indicating that only a fraction (~60%) of the cells displayed high levels of nuclear accumulation of fluorescent oligonucleotides after treatment with Lipofectin oligonucleotide complexes. A high level of cell-associated oligonucleotide is not sufficient *per se* to permit robust pharmacological effects. Thus, flow cytometry observations showed that the generation 3 dendrimer/antisense oligonucleotide complex provided a substantial increase in cellular association of oligonucleotide but failed to provide any inhibition of P-glycoprotein expression. By contrast, use of the GALA peptide had a minimal effect on total cellular accumulation of oligonucleotide, but the presence of GALA tended to enhance antisense inhibition of P-glycoprotein, although the result was not statistically signif-

icant. It is unclear why GALA had a very modest enhancing effect in the present studies, whereas in our previous work in another cell line, GALA seemed to be quite a promising adjuvant (Hughes *et al.*, 1996). Agents such as Lipofectin, GALA peptide and cationic facial amphiphiles presumably exert part of their effect by destabilizing endosomal membranes, thus allowing oligonucleotides to enter the cytoplasm (Akhtar and Juliano, 1992; Hughes *et al.*, 1996; Yu and Szoka, 1996). There seems to be substantial cell-type variations in the effectiveness of cationic lipid preparations and of amphipathic peptides such as GALA (current data *vs.* Hughes *et al.*, 1996) in the delivery of oligonucleotides to pharmacologically relevant sites.

There is substantial interest in the concept of using delivery systems to enhance the biological effectiveness of gene therapy as well as in antisense therapeutics. Clearly a number of delivery agents (or "adjuvants," as we termed them previously; Hughes *et al.*, 1996)) can be very useful in improving the effects of antisense compounds in the cell culture setting. At this point, little information is available about the toxicities, biodistribution patterns or pharmacological effectiveness of various potential "adjuvants' for nucleic acid therapy in the *in vivo* context. It will be important for the future evolution of gene therapy, as well as antisense therapeutics, to begin to address these difficult delivery issues.

Acknowledgments

The authors thank Prof. Steven L Regen of Lehigh University for providing samples of amphiphiles. The confocal microscope facility of the UNC Cystic Fibrosis Research Center was essential for this investigation.

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